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JUVENILE HORMONE BINDING PROTEINS IN THE HAEMOLYMPH OF THE INDIAN MEAL MOTH*

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Abstract—Binding of juvenile hormone (JH) to haemolymph proteins in larvae of the Indian meal moth, *Plodia interpunctella*, was measured by using radiolabeled *Hylaphora* JH. The proteins were separated by gel permeation chromatography and disc gel electrophoresis. A protein with an estimated molecular weight of $2{\cdot}5 \times 10^4$ selectively bound JH *in vitro*. Lipoproteins also bound the hormone but only when excess JH was present. We conclude that the lower mol. wt. JH binding protein serves as the actual carrier for JH in the larvae of this insect species.

INTRODUCTION

SEVERAL reports have suggested that lipoproteins serve to transport juvenile hormone (JH) as a water soluble complex from its site of synthesis in the corpus allatum to target tissues. TRAUTMANN (1972) first reported *in vitro* binding of two JH analogs by two lipoproteins in the haemolymph of *Tenebrio molitor* larvae. WHITMORE and GILBERT (1972) demonstrated, both *in vitro* and *in vivo*, that *Hylaphora* JH was selectively bound by a high density lipoprotein (*ca.* 2×10^5 mol. wt.) in the haemolymph of pupal and adult saturniid moths. Similar results were reported by EMMERICH and HARTMANN (1973) in adults of *Locusta migratoria*. In contrast, KRAMER *et al.* (1974) found a JH binding protein with a lower mol. wt. ($3{\cdot}4 \times 10^4$ mol. wt.) in larval haemolymph of *Manduca sexta* and indicated that the haemolymph lipoproteins formed a complex with the hormone only when the JH binding protein was saturated. We present similar evidence for a JH binding protein with an apparent mol. wt. of $2{\cdot}5 \times 10^4$ in larval haemolymph of the Indian meal moth, *Plodia interpunctella*.

MATERIALS AND METHODS

Test insects

Indian meal moth larvae were reared by the standardized procedure described by SILHACEK and MILLER (1972). Last instar larvae weighing 4, 8 or 20 mg/larva (representing the early, middle, and late parts of the instar, respectively) were selected from cultures for these experiments.

Collection of haemolymph

For each experiment, 0·1 ml of haemolymph was collected by clipping off a proleg and draining away the blood with a capillary tube. As the haemolymph was collected, it was mixed into 0·3 ml of a diluting medium containing 0·5 M sucrose, and $2{\cdot}5 \times 10^{-3}$M

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glutathione and buffered with 0·05 M Tris-HCl, pH 7·5. The diluting medium was held in an ice bath while the haemolymph was collected. Haemocytes were removed by centrifugation at $4{\cdot}7 \times 10^4$ g for 10 min at 4°C.

Labeling with ^{3}H-JH for gel permeation chromatography

Two to 36 µl, depending on the experiment, of 0·01 mM *Hylaphora*-C_{18} juvenile hormone [7-ethyl-1,2-^{3}H(N)], sp. act. 14·1 Ci/mM (New England Nuclear) dissolved in benzene–hexane, 4 : 1, was added to the 0·4 ml of diluted blood sample and the resulting emulsion was incubated at 22°C for 15 min. In one experiment, an unlabeled mixture of *Hylaphora* JH isomers (Hoffmann-LaRoche) ($3{\cdot}2 \times 10^{-3}$ M) was preincubated with haemolymph from 20-mg larvae prior to addition of ^{3}H-JH (5×10^{-6} M).

Chromatographic separation

One-tenth ml of haemolymph diluted to 0·4 ml was applied to a 0·9 cm i.d. × 60-cm long column of Sephadex G-200 equilibrated with 0·05 M Tris–HCl, pH 7·5 at 4°C. The proteins were then eluted with Tris–HCl buffer at a flow rate of 11 ml/hr. and 0·9 ml fractions were collected. Ammonium acetate–ammonium hydroxide buffer, 0·015 M ph 7·5, was substituted for the Tris–HCl buffer when the fractions were to be concentrated by lyophilization for disc gel electrophoresis. The elution patterns of the protein preparations were determined by monitoring the eluant at 280 nm using a UV absorption meter. Blue Dextran 2000 was used to determine the void volume. The apparent molecular weights of the resolved protein peaks were estimated by comparing with a calibration curve of the following protein standards: urease ($4{\cdot}82 \times 10^5$ mol. wt.), aldolase ($1{\cdot}58 \times 10^5$ mol. wt.), ovalbumin ($4{\cdot}5 \times 10^4$ mol. wt.), chymotrypsinogen A ($2{\cdot}5 \times 10^4$ mol. wt.), and ribonuclease A ($1{\cdot}37 \times 10^4$ mol. wt.) (GRANATH and KVIST, 1967).

The radiolabeling pattern of the resolved proteins was determined by mixing 0·2 ml of each fraction with 10 ml of scintillator solution (toluene, 500 ml; methyl cellusolve, 500 ml; PPO, 5·5 g; and POPOP, 300 mg) and counting the samples for 20 min in a Packard 3003 liquid-scintillation spectrophotometer. Quenching was monitored with an external standard.

Protein determination

Protein determinations were made according to the method of LOWRY *et al.* (1951).

Disc gel electrophoresis

Electrophoresis was performed at 4°C on 7% polyacrylamide gels at pH 8·9 with a current of 2 mA/gel by the method described by MAIZEL (1970). The lyophilized samples obtained from Sephadex G-200 fractionation were dissolved in 0·7 ml sucrose-ammonium acetate buffer and 0·3 ml was applied to each gel. The volume of the concentrating gel was proportionate to the sample volume. Proteins were stained with 0·25% Coomassie Brilliant Blue (R250) in 50% methanol that contained 7% acetic acid and destained overnight by diffusion in 5% methanol that contained 7% acetic acid. Lipoproteins were stained with Sudan Black B in acetone–acetic acid–water (20 : 15 : 80) overnight and destained with acetone–acetic acid–water (20 : 15 : 65) (MAUER, 1971). Unstained gels were sliced into 1-mm sections and prepared for liquid scintillation counting according to the method of MUTO (1968).

In one experiment, 0·1 ml of haemolymph collected from 4-mg larvae was diluted with 0·2 ml sucrose-glutathione buffer solution, centrifuged, and then incubated for 15 min at 37°C with $5{\cdot}6 \times 10^{-6}$ M unlabeled JH and 3×10^{-7} M ^{3}H-JH simultaneously. After incubation, 200 µg of protein was applied per gel.

Thin-layer chromatography

JH and its metabolites were tentatively identified by TLC (WHITE, 1972; AJAMI and RIDDIFORD, 1973; and SLADE and ZIBITT, 1971). Polyacrylamide gel slices and haemolymph

fractions were extracted with chloroform–methanol (2 : 1) by the method of FOLCH *et al.* (1957). The extracts were then chromatographed in three different solvent systems on precoated silica gel plates (Quantum Industries). The thin layer plates were fractionated with an automatic zonal scraper, and the radioactivity in the resultant fractions was determined by liquid scintillation counting.

RESULTS

Figure 1 shows the radioactivity and elution patterns of haemolymph from fifth-instar larvae (4 mg/larva) that contained $2{\cdot}5\times10^{-8}$ M ^{3}H–JH. Two major UV absorbing peaks were resolved. The apparent molecular weight of proteins in the first peak eluted from the column was estimated at $5{\cdot}8\times10^5$. The slower eluting peak corresponded to a mol. wt. that was less than that of ribonuclease ($1{\cdot}36\times10^4$).

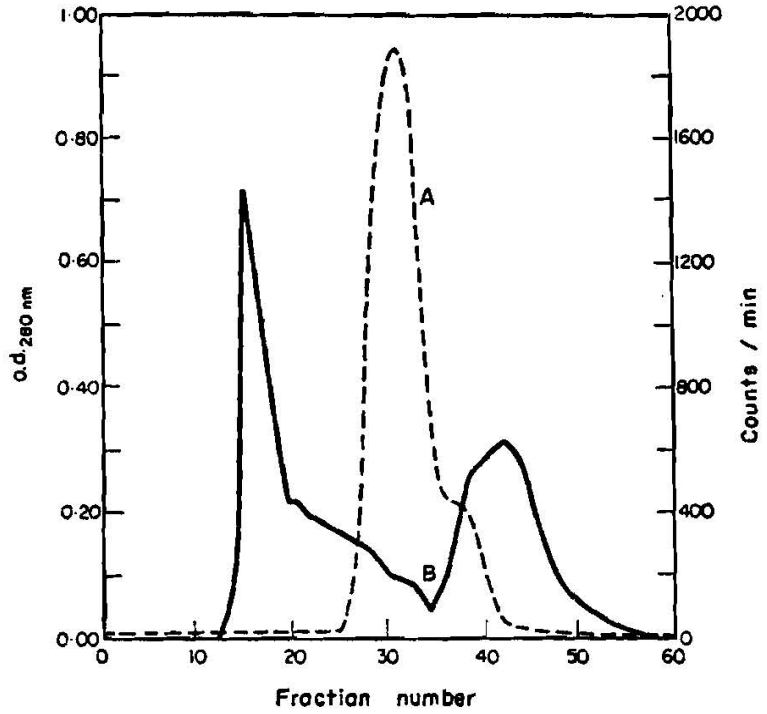

FIG. 1. Elution pattern on Sephadex G-200 of 0·1 ml haemolymph from fifth-instar larvae of *Plodia* (4-mg av. wt/larva) incubated with ^{3}H–JH ($2{\cdot}5\times10^{-8}$ M) for 15 min at 20°C. A, radioactivity; B, absorbance at 280 nm. See methods for experimental details.

The radioactivity eluted between the two major 280-nm absorption peaks; the protein associated with this radioactivity had an estimated mol. wt. of $2{\cdot}5\times10^4$. A shoulder of the radioactive peak also overlapped the low molecular weight absorption peak. Of the radioactivity applied to the column with the haemolymph, 77·6% was recovered in the first 60 fractions. When emulsified ^{3}H–JH alone was applied, only 5·5% of the radioactivity was recovered in the same volume. The free hormone did not elute off the column in a single peak but washed off gradually, an indication that the counts that coincided with the absorption peaks represented bound hormone.

The next question was whether similar elution patterns of label and protein could be obtained with older insects. Haemolymph samples from 8- and 20-mg larvae (just prior to pupation) were incubated with $2{\cdot}5\times10^{-8}$ M ^{3}H–JH and applied to a Sephadex G-200 column in two separate tests. The major radioactive peak eluted between the two absorption peaks with the 8-mg larvae as shown in Fig. 2. A similar pattern was obtained with haemolymph from 20 mg larvae (Fig. 3). The protein associated with this radioactivity in both stages had an estimated mol. wt. of $2{\cdot}5\times10^4$. Radioactivity also overlapped the low molecular weight peak (Figs. 2 and 3).

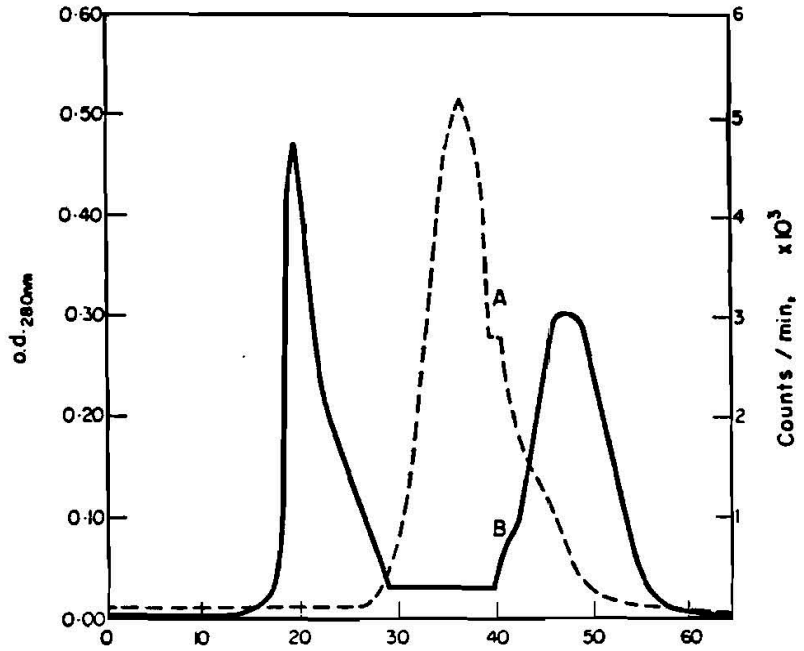

Fig. 2. Elution pattern on Sephadex G-200 of 0·1 ml of haemolymph from fifth-instar larvae of *Plodia* (8-mg av. wt/larva) incubated with ^{3}H–JH ($2{\cdot}5\times10^{-8}$ M) for 15 min at 20°C. A, radioactivity; B, absorbance at 280 nm. See methods for experimental details.

Since the possibility existed that the major peak of radioactivity represented metabolized JH, we incubated blood from 8 mg larvae with $1{\cdot}1\times10^{-7}$ M ^{3}H–JH and applied the sample to a G-200 column. Fractions that contained the radioactive peak were combined into two groups (I, fractions 32–40 and II, fractions 44–47), extracted with chloroform–methanol (2 : 1), and analyzed by thin layer chromatography (Fig. 4). Over 95% of the radioactivity associated with the $2{\cdot}5\times10^4$ mol. wt. protein (Group I fractions) was intact JH; the remainder had been metabolized to the dihydroxy methyl ester. However, in Group II, only 50% of the radioactivity was JH; over 40% had metabolized to the dihydroxy methyl ester and the remainder to the dihydroxy acid.

Disc gel electrophoresis

The nature of the radiolabeled proteins, was determined by incubating haemolymph from 4-mg larvae with $3{\cdot}6\times10^{-7}$ M ^{3}H–JH plus $3{\cdot}4\times10^{-3}$ M

ERRATUM

Should read: "...over 40% had been metabolized to the expoxy acid and the remainder to the dihydroxy acid."

unlabeled *Hylaphora* JH and then chromatographing the incubate on a Sephadex G-200 column. In contrast to the earlier results obtained when low JH levels were used (Fig. 1), incubation with high JH levels resulted in an additional peak that eluted with the $5{\cdot}8 \times 10^5$ mol. wt. proteins (Fig. 5). Also, the elution volume of the main radioactive peak (fraction 39, 35 ml; Fig. 5) was greater than that in the earlier test (fraction 30, 28 ml; Fig. 1).

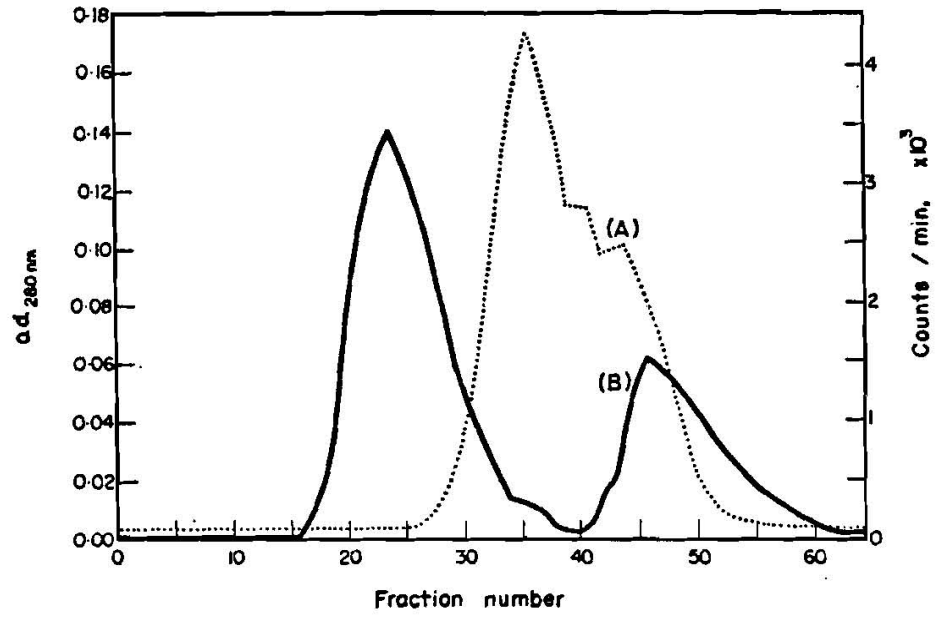

FIG. 3. Elution pattern on Sephadex G-200 of 0·1 ml haemolymph from fifth-instar larvae of *Plodia* (20-mg av. wt/larva) incubated with ^{3}H–JH ($2{\cdot}5 \times 10^{-6}$ M) for 15 min at 20°C. A, radioactivity; B, absorbance at 280 nm. See methods for experimental details.

The fractions containing the three radioactive maxima (Fig. 5) were combined into three groups (I, 11–21; II, 28–41; III, 42–56) for analysis by polyacrylamide gel electrophoresis. In Group I, the tritium label was associated with two lipoprotein bands near the origin, with a dark-staining JH-carrying band midway down the gel, and with the bromophenol blue front (Fig. 6). In Group II, fewer counts were associated with the lipoprotein bands and the bromophenol blue front, but more was bound by the JH-carrying band. In Group III, counts were present only at the bromophenol blue front. These data indicated that the Group II proteins (Fig. 4 and 5) that contained over 95% unaltered JH also contained the carrier protein. However, considerable radioactivity was obviously lost from this fraction during lyophilization and electrophoresis. It is not known whether this loss resulted from JH breakdown, volatilization, or both. Comparison of Coomassie blue and Sudan black staining (Fig. 6, A and B) of the electrophoretic gels clearly indicates that the suspected JH-carrier protein is not a lipoprotein. It should be emphasized that the radioactivity associated with the lipoprotein bands (Fig. 6) only occurs when JH is present in large excess.

An additional experiment was conducted to further demonstrate whether the label associated with the protein bands separated by disc gel electrophoresis represented JH or its metabolities. Whole haemolymph from 4-mg larvae was incubated with 3×10^{-7} M ^{3}H-JH plus $5{\cdot}6\times10^{-6}$ M cold JH and electrophoresed. The gel was then cut into three sections: the first 10 mm from the origin contained the lipoproteins, the next 20 mm contained the JH binding band, and the remainder of the gel contained the bromophenol blue band. Each of the three sections of the gel was extracted with chloroform–methanol (2 : 1) and analyzed by thin layer chromatography. Sixty-nine per cent of the label associated with the first 10-mm section was JH; the remaining count was tentatively identified as 22·7% dihydroxy

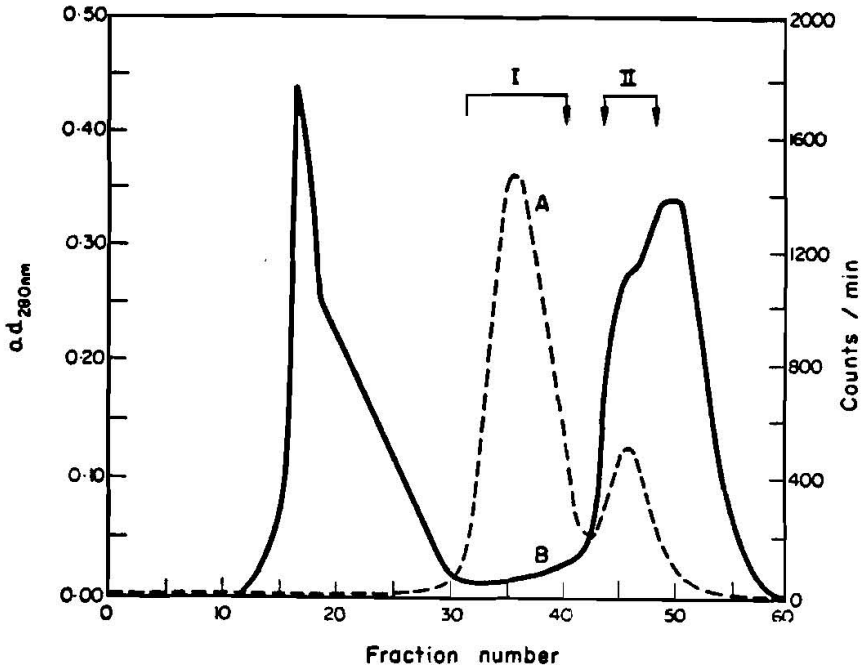

FIG. 4. Elution pattern on Sephadex G-200 of 0·1 ml of haemolymph from fifth-instar larvae of *Plodia* (8-mg av. wt/larva) incubated with $1{\cdot}1\times10^{-7}$ M ^{3}H-JH for 15 min at 20°C. A, radioactivity; B, absorbance at 280 nm. Combined fractions in group I and II were analyzed by thin layer chromatography. See methods for experimental details.

methyl ester, 5·5% epoxy acid, and 1·5% dihydroxy acid. In the next 20-mm section that contained the JH-binding band, over 90% of the radioactivity was JH; the remainder was divided between the dihydroxy methyl ester and epoxy acid. In the remainder of the gel, the label was primarily associated with metabolities of JH: only 4·3% was JH, and 3·2, 37·0 and 48·8% was dihydroxy methyl ester, epoxy acid, and dihydroxy acid, respectively. These data indicated that the majority of the count associated with the lipoproteins and the JH-binding band was JH; the label running with the bromophenol blue band was associated with the metabolities of JH. However, identification of JH and its metabolites by

chromatographic behavior data alone is only tentative and still requires a more rigorous chemical confirmation.

Since labeling of the lipoproteins occurred when the JH concentration was excessively high, it appeared that at high concentrations of JH the carrier protein ($2{\cdot}5\times10^4$ mol. wt.) became saturated and allowed JH to bind nonspecifically to lipoprotein. This hypothesis was tested by taking haemolymph from 20-mg larvae and preincubating it with $3{\cdot}2\times10^{-2}$ M unlabeled *Hylaphora* JH for 15 min at 22°C. Then ^{3}H–JH was added to give a final concentration of 5×10^{-8} M ^{3}H–JH

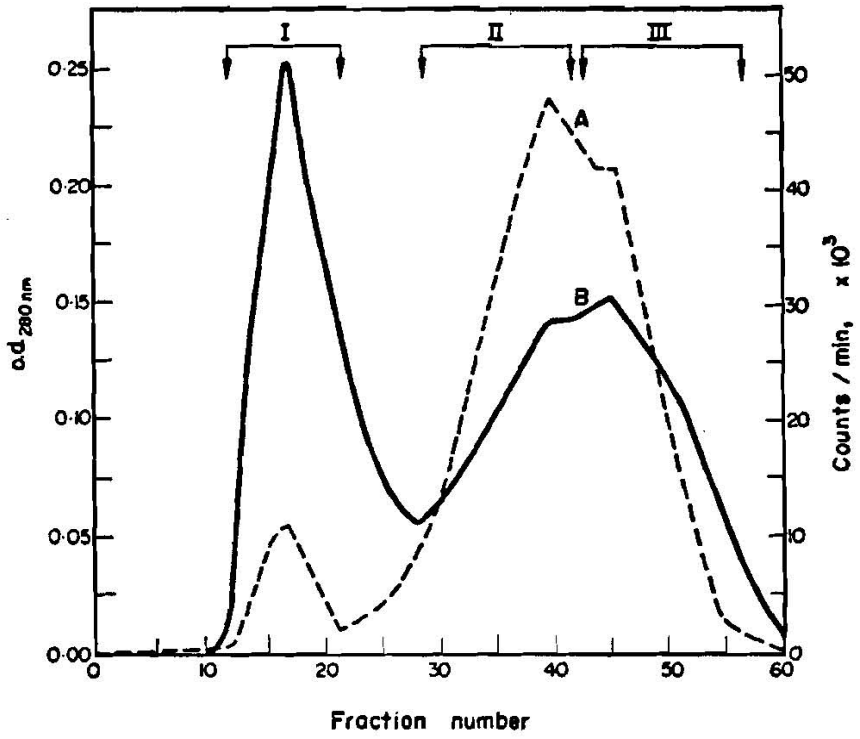

FIG. 5. Elution pattern on Sephadex G-200 of 0·1 ml haemolymph from fifth-instar larvae of *Plodia* (4-mg av. wt/larva) incubated with ^{3}H–JH ($3{\cdot}6\times10^{-7}$ M) plus $3{\cdot}4\times10^{-3}$ M cold JH for 15 min at 20°C. A, radioactivity; B, absorbance at 280 nm. Combined fractions in Groups I, II and III were analyzed by polyacrylamide gel electrophoresis. See methods for experimental details.

and incubated for an additional 15 min. Haemolymph proteins were then fractionated on Sephadex G-200. Preincubation with unlabeled JH before addition of the ^{3}H–JH eliminated the radioactive peak associated with $2{\cdot}5\times10^4$ mol. wt. proteins which resulted when only ^{3}H–JH was incubated with the haemolymph (Fig. 7). More important was the appearance of a radioactive peak coinciding with the lipoproteins in the first peak eluted off the column. These results indicated that the unlabeled JH saturated the binding sites on the $2{\cdot}5\times10^4$ mol. wt. JH carrier protein which minimized subsequent binding and exchange. Presumably, the lipoproteins were either not saturated with unlabeled JH or they exchanged JH more readily because of their nonspecificity in binding the JH molecule.

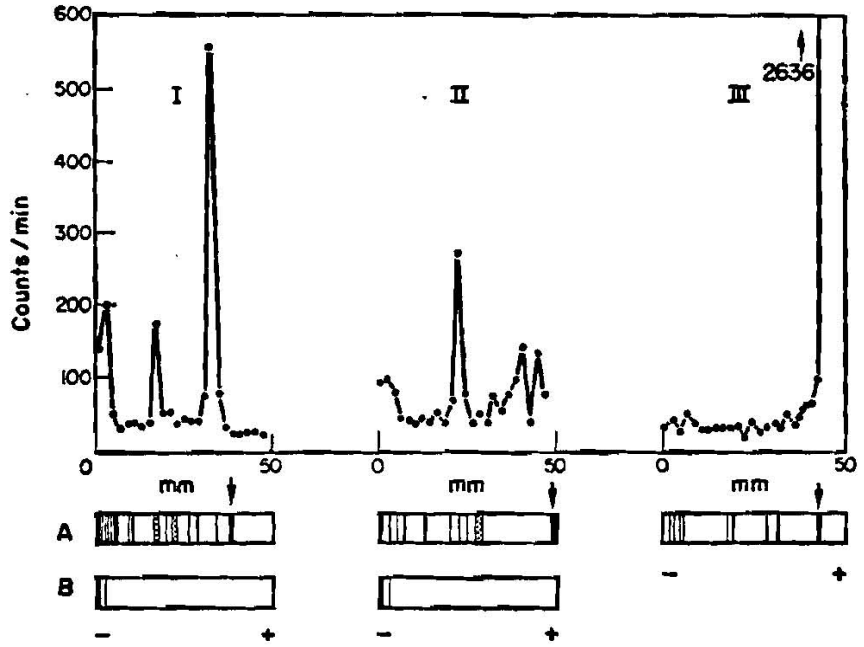

FIG. 6. Disc gel electrophoretic and radioactivity patterns of combined fractions in Groups I (fractions 11–21), II (fractions 28–41), and III (fractions 42–56) obtained from filtration of haemolymph from *Plodia* larvae on Sephadex G-200 (See Fig. 5). A, Coomassie blue; B, Sudan black. Arrow indicates bromophenol blue front. See methods for experimental details.

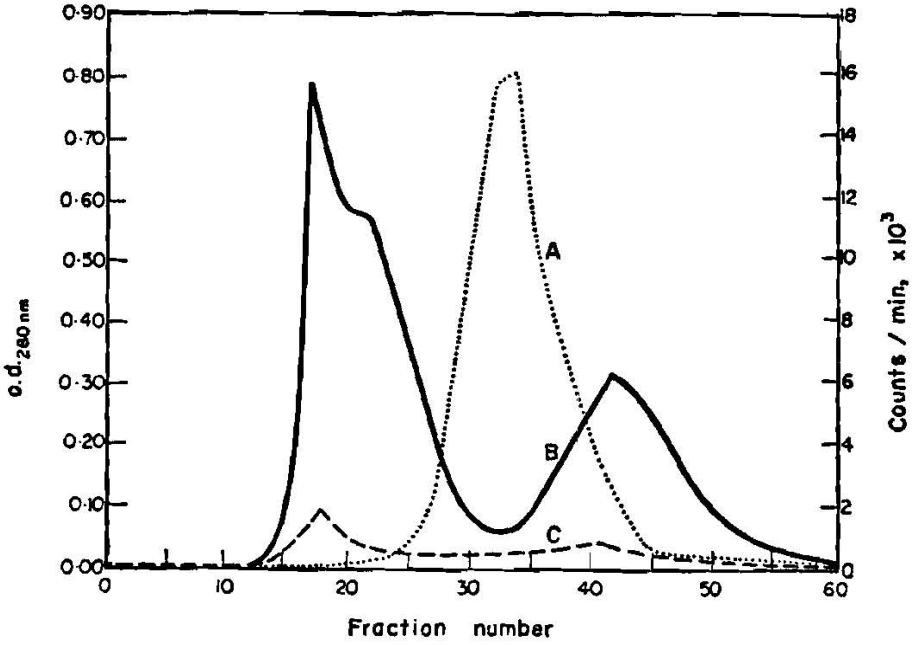

FIG. 7. Elution pattern of 0·1 ml haemolymph from fifth-instar larvae of *Plodia* (20-mg av. wt/larva) on Sephadex G-200. A, haemolymph incubated with ^{3}H-JH (5×10^{-8} M) alone; B, absorbance at 280 nm; C, haemolymph preincubated with cold *Hylaphora* JH ($3{\cdot}2 \times 10^{-3}$ M) for 15 min at 20°C prior to incubation with ^{3}H-JH (5×10^{-8} M) for 15 min at 20°C. See methods for experimental details.

DISCUSSION

Several investigators have reported the significance of lipoproteins in transporting JH in the haemolymph of insects (WHITMORE and GILBERT, 1972; TRAUTMANN, 1972; EMMERICH and HARTMANN, 1973). Recently, KRAMER *et al.* (1974) found a JH carrier protein ($3{\cdot}4 \times 10^4$ mol. wt.) to selectively bind JH in the haemolymph of *Manduca sexta*. Lipoproteins in this insect were also found to bind JH but only when the hormone was in excess and the carrier protein was saturated. Our results were similar to those of KRAMER *et al.* (1974) in that JH was selectively bound by a protein with a mol. wt. tentatively estimated at $2{\cdot}5 \times 10^4$. Our data also indicate that lipoproteins bind JH (and perhaps the dihydroxy metabolite) when the binding sites for JH on the carrier protein are saturated and JH is in excess. The exchange of ^{3}H–JH for unlabeled JH by the lipoproteins indicated the formation of a lipoprotein-JH complex with low binding energy and/or a high number of binding sites that were not initially saturated with unlabeled JH. The absence of exchange of ^{3}H-JH for unlabeled JH by the $2{\cdot}5 \times 10^4$ mol. wt. protein suggests a tight binding in this complex. Whether a similar type and size of protein serves to transport the hormone in adults of the Indian meal moth remains to be determined.

WHITMORE and GILBERT (1972) working with saturniid moth pupae and adults and EMMERICH and HARTMANN (1973) working with adult locusts reported molecular weights of about 2×10^5 for the JH-carrier protein. In view of our results and those of KRAMER *et al.* (1974), this binding of JH to high molecular weight proteins could result from nonspecific binding of labeled JH to lipoproteins. A lowered titer of JH carrier protein in the pupa or an endogeneously saturated JH carrier protein in the adult would result in appreciable binding of added JH to lipoprotein. On the other hand, lipoproteins may serve as a JH-carrier during all developmental stages in some insect species, while in other species lipoproteins may function as a JH carrier only after pupal–adult apolysis or not at all. The interrelationships of lipoprotein and the low molecular weight carrier protein at different developmental stages are currently being investigated.

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